Journal of Experimental Marine Biology and Ecology 381 (2009) 1–9

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Nutritional value of the cryptophyte *Rhodomonas lens* for *Artemia* sp.

Pedro Seixas, Paula Coutinho, Martiña Ferreira, Ana Otero *

Dpto. de Microbiología y Parasitología, Facultad de Biología — Edifício CIBUS, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, A Coruña, Spain

ARTICLE INFO

Article history:
Received 15 May 2009
Received in revised form 1 September 2009
Accepted 2 September 2009

Keywords:
Artemia
Cryptophyte
Growth
Microalgae
Rhodomonas
Tetraselmis

ABSTRACT

Juvenile or adult *Artemia* sp. are often used as live prey for the rearing of early life stages of some crustacean, fish and cephalopod species. The improvements of both *Artemia* growth and its biochemical composition are key issues for the suitable use of *Artemia* biomass in these rearing processes. In this study we evaluated the growth and survival rates of *Artemia* fed with the cryptophyte *Rhodomonas lens* in comparison with different microalgal species commonly used in aquaculture: the prasinophyte *Tetraselmis suecica*, the prymnesiophyte *Isochrysis galbana* Parke, and the eustigmatophyte *Nannochloropsis gaditana*. Microalgae were cultured semi-continuously in nutrient saturated conditions and with a daily renewal rate of 30% of the volume of cultures, to obtain biomass of controlled and optimized composition. Considerable differences in *Artemia* growth were observed, as well as in the survival rate. At day 8 of rearing, *Artemia* fed *R. lens* had the highest length (4.9 ± 0.6 mm, $P<0.001$), followed by individuals fed *T. suecica* (4.2 ± 0.7 mm), *I. galbana* (3.6 ± 0.7 mm) and finally those fed *N. gaditana* (1.5 ± 0.2 mm). The survival rate of *Artemia* fed *N. gaditana* (18 ± 3%) was much lower ($P<0.001$) than values found for the remaining groups (69 to 88%). The growth rate of *Artemia* obtained with *R. lens* was in general much higher than with other microalgal diets previously reported in the literature. The higher protein content of *R. lens* could explain the higher growth obtained with this species, but differences of *Artemia* growth with the different diets could not be explained solely on the basis of the gross composition of microalgae. Factors such as cell size and digestibility all seem to contribute to the results observed. Another trial was carried out to investigate differences in *Artemia* growth and on its biochemical composition when fed the best two diets: *R. lens* or *T. suecica*. The fatty acid (FA) and total amino acid (AA) composition of both microalgal species and the composition of *Artemia* were assessed as well. As found in the first experiment individuals fed *R. lens* (group ARHO) grew faster than those fed *T. suecica* (group ATET), attaining 3.6 ± 0.3 mm and 3.2 ± 0.4 mm ($P<0.001$), respectively, after 5 days of rearing. The much higher AA content obtained in *R. lens* may be on the basis of the higher growth obtained with this species. Protein and carbohydrate levels in *Artemia* juveniles were very similar in both groups (64–68% of dry weight, and 8–10%, respectively). Lipid was slightly lower in ARHO (12%) than in ATET (15%, $P<0.01$). Regarding the FA composition, juveniles from group ARHO contained higher levels of eicosapentaenoic acid (EPA, 6.2%) than juveniles from ATET (4.1%, $P<0.01$), whereas docosahexaenoic acid (DHA) was only found in juveniles from ARHO (1.1%). Taking into account that the daily productivity of *R. lens* culture was higher than, or at least equal, the remaining microalgal species this cryptophyte is confirmed as an excellent diet to optimize the growth of *Artemia*, as well as to improve its biochemical composition.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Microalgae are the basis of the food chain in many aquaculture processes. They are used to directly feed all life stages of filter-feeder molluscs (Enright et al., 1986; Brown et al., 1997) and larval or juvenile stages of some fish and crustacean species (Reitan et al., 1997; Piña et al., 2006); or indirectly to feed/enrich copepods, rotifers and *Artemia*, which in turn are commonly used as major live prey for the rearing of many marine larval species (Støttrup and Jensen, 1990; Dhert et al., 2001; Sorgeloos et al., 2001; Støttrup, 2003; Aragão et al., 2004). Despite newly hatched nauplii and/or 24 h-enriched nauplii being the most common form of *Artemia* used in aquaculture (Sorgeloos et al., 2001), juvenile or adult *Artemia* are also utilized to rear early life stages of some crustacean species (Dhert et al., 1993; Conklin, 1995; Ritar et al., 2003; Tlusty et al., 2005), fishes (Lim et al., 2001; Woods, 2003) and cephalopods (Domingues et al., 2001; Iglesias et al., 2007). Different kinds of diets are frequently used for the on-growing of *Artemia* such as live microalgae, dried algae, bacteria and/or yeast and waste products from food industry, but best yields are undoubtedly obtained with live microalgae (Dhont and

* Corresponding author. Tel.: +34 981 563100x16913/14944; fax: +34 981 528006.
E-mail address: anamaria.otero@usc.es (A. Otero).

0022-0981/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jembe.2009.09.007

Lavens, 1996). The selected microalgal species is a crucial issue for the improvement of *Artemia* growth, modifying both, its growth rate and its biochemical composition. Despite the difficulties in the direct comparison of *Artemia* growth results among several works, due to the diversity of culture conditions of both the microalgae and *Artemia*, a summary of some published works on this subject is shown in Table 1. *Tetraselmis suecica* has been repeatedly reported as one of the best species for the on-growing of *Artemia* (Table 1). Nevertheless, an undefined species of *Cryptomonas* produced best growth of *Artemia* nauplii in only 24 h and after 7 days of rearing, when compared to other standard microalgae such as *Tetraselmis* sp., *Isochrysis* aff. *galbana* T-ISO or *Chaetoceros* sp. (Thinh et al., 1999), which indicate the need to explore the nutritional potential of Cryptophyte species. We have recently shown that another Cryptophyte, *Rhodomonas lens*, produced excellent results in the growth and composition of *Artemia* metanauplii enriched for 26 h, in comparison with other microalgal species such as *T. suecica*, *I. galbana* Parke and *I.* aff. *galbana* T-ISO (Seixas et al., 2008). *Rhodomonas* sp. has also been previously shown to constitute a high-quality diet to rear calanoid copepods (Koski et al., 1998), and was recently confirmed as an excellent microalgal diet for the development of the copepod *Acartia sinjiensis* (Knuckey et al., 2005). It is therefore interesting to test the nutritional value of this species for *Artemia* sp. in comparison with other species under controlled conditions.

One of the main problems encountered in the interpretation of results from culture experiments comparing the nutritional value of different microalgal species is the lack of control of the biochemical composition of the microalgal biomass. Most feeding experiments have been carried out using microalgal batch cultures in which the biochemical composition of the microalgae is not stable and differences in the growth rate of the microalgal species make direct comparison of their nutritional value difficult. Moreover, the batch cultures generally used in aquaculture offer low productivity, the level of contaminant bacteria is high, and cultures are harvested near the stationary phase, that is when the nutritional composition of microalgae is far from optimal (Fábregas et al. 1996; Flynn et al., 1996; Otero et al., 2002). In contrast, continuous and semi-continuous cultures of microalgae produce biomass of constant and controlled biochemical composition, and have long been demonstrated to noticeably improve the growth and development of filter-feeders such as rotifers and *Artemia* (Scott, 1980; Taub, 1980, Fábregas et al. 1996; 1998), and to boost their nutritional value even in short-time enrichments (Fábregas et al., 2001; Ferreira et al., 2008). Therefore, they constitute an exceptional tool for the evaluation of the nutritional value of different microalgal species. In the case of *Artemia*, previous works showed that growth and survival of *Artemia* was superior in groups receiving *T. suecica* cultured semi-continuously in nutrient saturated conditions (Fábregas et al., 1996). Moreover, important changes in the gross biochemical composition of

Table 1
Summary of some works reporting *Artemia* sp. growth with different microalgal species.

Authors	Microalgae species used to feed *Artemia* sp.	Culture method and nutrient concentration	Temperature and days of rearing	Best microalgal diet	Length of *Artemia* sp.
Sick (1976)	Five species tested: *Chlamydomonas sphagnicola*, *Dunaliella viridis*, *Chlorella conductrix*, *Platymonas elliptica*, *Nitzschia closterium*	Batch, harvest of 120 h-old algae No information about nutrient medium	25 °C 16 days	*C. sphagnicola* *D. viridis*	1.8 mm (day 6) 8.9 mm (day 16) 2.9 mm (day 6) 5.7 mm (day 16)
Abreu-Grobois et al. (1991)	*Dunaliella tertiolecta*	Batch, harvest of cells with no more than 5 days. No information about nutrient medium	28 °C 14 days		10.0 mm (day 14)
Gamallo (1992)	Six species: *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Dunaliella tertiolecta*, *Isochrysis galbana* Parke, *Nannochloris atomus*, *Nitzschia acicularis*	Batch Medium ALGAL 2 mM N l^{-1}	25 °C 23 days	*T. suecica* *D. tertiolecta*	4.6 mm (day 8) 4.4 mm (day 8)
Fábregas et al. (1996)	*Tetraselmis suecica*	Semi-continuous with daily renewal rate of 50%. ALGAL 8 mmol N l^{-1}	25 °C 19 days		8.3 mm (day 19)
Evjemo and Olsen (1999)	*Isochrysis galbana* T-ISO	Semi-continuous culture *f*/2 medium	26–28 °C 12 days		3.3 mm (day 6) 5.9 mm (day 12)
García-Ulloa et al. (1999)	*Tetraselmis suecica* *Chaetoceros calcitrans* *Spirulina* sp. (dried)	Semi-continuous Daily harvest of a partial volume *f*/2 medium	25.5 ± 2.5 °C 10 days	*T. suecica* *Spirulina* sp. (dried)	2.1 mm (day 6) 4.9 mm (day 10) 3.2 mm (day 6) 4.7 mm (day 10)
Naegel (1999)	*Chaetoceros* sp.	Not reported	25.0 ± 0.2 °C 11 days		2.0 mm (day 6) 4.6 mm (day 11)
Thinh et al. (1999)	Thirteen species of tropical Autralian microalgae (benthic and planktonic)	Batch, harvest at the end of log-phase *f* medium	25 °C 7 days	*Cryptomonas* sp. *Chaetoceros* sp. *Tetraselmis* sp. *Cryptomonas* sp. *Chaetoceros* sp.	0.92 (day 1) 0.89 (day 1) 0.88 (day 1) 6.5 mm (day 7) 5.5 mm (day 7)
Godínez et al. (2004)	*Tetraselmis suecica* *Chaetoceros muelleri*	Semi-continuous with daily harvest of a partial volume. *f*/2 medium	25 ± 1 °C 10 days	*T. suecica* *C. muelleri*	4.5 mm (day 10) 3.7 mm (day 10)
Lora-Vilchis et al. (2004)	*Chaetoceros muelleri* *Isochrysis galbana* T-ISO	Semi-continuous with daily renewal rate of 25%. *f* medium	27.5 ± 0.5 °C 7 days	*C. muelleri* *I. galbana* T-ISO	6.0 mm (day 7) 4.2 mm (day 7)
Marques et al. (2004)	*Dunaliella tertiolecta* (two strains: 19/6B; 19/27) *Tetraselmis suecica* (two strains: 66/4; 66/22A)	Batch, harvest in the middle of exponential growth or in stationary (both conditions) Walne medium	28 °C 6 days	*T. suecica* 66/4 *T. suecica* 66/22A *D. tertiolecta* 19/6B	3.5 mm (day 6) 4.0 mm (day 6) 3.2 mm (day 6)
Present work (two experiments)	*Tetraselmis suecica*, *Rhodomonas lens*, *Nannochloropis gaditana*, *Isochrysis galbana* Parke	Semi-continuous with daily renewal rate of 30%. Nutrient saturated (2 or 4 mM N l^{-1})	26.5 ± 0.5 °C 5 and 8 days	*R. lens*	3.6 mm (day 5) 4.9 mm (dia 8)

Data of *Artemia* sp. length at different days is shown according to information reported by authors and with relevance to the present work.

adult *Artemia* were found when feeding individuals with *T. suecica* cultured semi-continuously at different daily renewal rates (Fábregas et al., 2001).

In the present study we compared the growth of *Artemia* fed either *R. lens* or other microalgal species widely used in aquaculture: *T. suecica*, *I. galbana* Parke and *Nannochloropsis gaditana*, all cultured semi-continuously under nutrient saturated conditions. Since the nutritional composition of *Artemia* is also a main concern when feeding marine larval species, we also analysed the biochemical composition of the microalgal diets producing the best growth results and of the *Artemia* juveniles obtained.

2. Material and methods

2.1. Microalgae cultures

The marine microalgae *T. suecica* Kylin, *I. galbana* Parke (both isolated from Ría de Arousa, Spain), *R. lens* Pascher et Ruttner (CCMP 739) and *N. gaditana* (CCMP 527), were grown semi-continuously in 6 l glass flat-bottom flasks containing 5 l of sterilized sea water (salinity of 35ppt), being submitted to 12 h:12 h light/dark cycle periods and an irradiance of 197 μmol photon m^{-2} s^{-1} in the rear and of 166 μmol photon m^{-2} s^{-1} from below. Irradiance was measured with a luxmeter Neurtex HD8366 followed by conversion according to the formula proposed by Ginzburg (1987). The pH of cultures was kept below 8.0 through the periodic injection of CO_2 during the light period. Cultures were started in batch mode with a nutrient concentration of 4 mM N l^{-1} (Fábregas et al., 1984), and daily dilutions at a renewal rate of 30% of the volume of cultures were carried out once cultures approached late-logarithmic phase, with water enriched with the same nutrient concentration for *T. suecica* and *R. lens* and with 2 mM N l^{-1} for *I. galbana* and *N. gaditana*. These nutrient concentrations ensured nutrient saturation for the conditions applied, which was confirmed by spectrophotometric determination of the remaining NO_3^{-2} concentration (Clesceri et al., 1989) in the harvested cultures. Once the steady state was achieved, as assessed by steady cell density, the daily harvested cultures were used to feed *Artemia* nauplii. Cell density was measured by means of a Neubauer haemacytometer. Microalgae dry weight was determined by filtering 2 ml of the harvested biomass through carbonised Whatman GF/C glass fibre filters (Whatman, Brentford, UK). Filters were washed twice with ammonium formiate 0.5 M before being dried at 80 °C for 24 h. Microalgal biomass was harvested on different days during the steady state, centrifuged and immediately frozen at −18 °C for biochemical analysis.

2.2. Experiments of Artemia sp. growth

Artemia cysts (AF, INVE, Dendermonde, Belgium) were hatched in seawater adjusted to a salinity of 30 ppt, constant aeration and water temperature of 28 ± 1 °C. On day 0 newly hatched nauplii were rinsed with sterilized sea water and transferred in triplicate to glass flasks containing 700 ml of sterilized seawater (30 ppt). Nauplii rearing conditions were as follows: initial density of 2.0 nauplii ml^{-1}, constant aeration provided by capillary tubes, temperature of 26.5 ± 0.5 °C and dim light for 24 h. Initial food ration was established as 25 μg dry weight (DW) of microalgae per nauplii, equivalent to the following doses: 125×10^3 cells of *T. suecica*, assuming a cellular DW of 200 pg $cell^{-1}$; 250×10^3 cells of *R. lens* (DW of 100 pg $cell^{-1}$); 1.0×10^6 cells of *I. galbana* (DW of 25 pg $cell^{-1}$); and 3.1×10^6 cells of *N. gaditana* (DW of 8.0 pg $cell^{-1}$). The amount of food was increased gradually as *Artemia* individuals were growing, depending on the transparency of the culture media, so that almost all food supplied was ingested (Sorgeloos et al., 1986). Water was completely renewed every 2 days to remove *Artemia* faeces and old microalgae cells that could remain in the water.

In a first experiment *Artemia* nauplii were grown for 8 days for the comparison between *R. lens* (ARHO) and three other species commonly used in aquaculture: *T. suecica* (ATET), *I. galbana* (AISO) and *N. gaditana* (ANANO). In a second experiment *Artemia* nauplii were grown for 5 days only with *T. suecica* or *R. lens*, in order to address the nutritional composition of *Artemia* juveniles as well. On the fifth day of culture, food was supplied for further 4 h to ensure maximum enrichment of *Artemia* prior to its sampling for biochemical composition analysis. The total length of *Artemia* individuals was measured under a stereoscope using a calibrated ocular micrometer (25 *Artemia* per replicate), whereas final dry weight was calculated by weighing samples of 20 individuals ($n = 5$) per replicate, which were washed with distilled water and dried at 101 ± 1 °C for 24 h. Feed conversion rate (FCR) was calculated as follows: total food supplied (dry weight of microlgae)/ biomass of *Artemia* obtained (dry weight). Survival was recorded on the second day and at the end of the experiments.

2.3. Biochemical composition analysis

Protein content was determined by the Folin-phenol method (Lowry et al., 1951), after hydrolysis with NaOH 1.0 M at 95 °C; carbohydrates by the phenol/sulphuric acid method (Kochert, 1978) and lipids were quantified by the charring method (Marsh and Weinstein, 1966) after extraction of total lipids (Bligh and Dyer, 1959). Phycoerythrin was extracted from *R. lens* by re-suspending 3 ml culture-samples previously centrifuged in distilled water, followed by frosting at −20 °C to disrupt cells. Determination of phycoerythrin was carried out using the formulas proposed by Bennet and Bogorad (1973) after reading pigment concentrations in a spectrophotometer at wavelengths of 565 nm, 620 nm and 650 nm (Bryant et al., 1979). C–N–H of microalgae was determined with an elemental autoanalyser (Fisons 1108) on freeze-dried samples. Total fatty acids were identified and quantified using a gas chromatograph–mass spectrograph (GC–MS Fisons Instruments, MD-800, Beverly, Mass.), equipped with an Omegawax™ 250 column 30 m × 0.25 mm (Supelco, Inc.), after methanolysis of the lipid extracts with 5% HCl in methanol at 85 °C during 2:30 h, and extraction with hexane (Sato and Murata, 1988). Triheptadecanoin (Sigma®, St. Louis, Mo.) was used as internal standard. Caloric values were calculated using the conversion formulas suggested by the National Research Council (1993). Total amino acids of *T. suecica* and *R. lens* were determined in 17.5 mg of centrifuged cultures. Samples were hydrolysed in 25 ml of HCl 6.0 M at 105 °C for 24 h. The obtained hydrolyzed solutions and amino acid standards (Waters, Standards WAT 088122) were derivatized using AccQ-Tag® System for amino acid analysis (Water, Milford, MA) and run on a modification of the reversed-phase HPLC system (Waters Associates). A reverse-phase column (AccQ-Tag, 150 mm long, 3.9 mm internal diameter) was used for the separation of the different amino acids. Samples of 10 μl were injected by auto sampler, and the eluting products were measured with a fluorescent detector at excitation wavelengths of 250 nm and 395 nm. Chromatograms were recorded using the software program Breeze. Results for tryptophan are not reported since this amino acid is destroyed by acid hydrolysis. All the analyses were carried out in triplicate, except for total amino acids for which a single analysis (double injection in HPLC) was done.

2.4. Statistical analysis

Statistical analyses were done using the software SPSS V 15.0.1 statistical package (SPSS, Inc.). Data of *Artemia* length were checked for requirements of normality (Kolmogorov–Smirnov test). For comparisons of dry weight and *Artemia* length among groups, analysis of variance (ANOVA) followed by Tukey–Kramer HSD tests for post-hoc multiple comparisons were carried out ($\alpha = 0.05$). Comparisons of data between groups of experiment two were carried out by student's *t*-test. Percentages of survival and biochemical composition were arcsine-√ transformed before statistical analysis (Zar, 1999).

3. Results

3.1. Productivity and gross biochemical composition of microalgae

The steady state densities (cells ml^{-1}) attained in the different microalgal cultures in the course of the experiment increased as follows: *T. suecica* ($2.0 \pm 0.2 \times 10^6$) < *R. lens* ($3.7 \pm 0.2 \times 10^6$, $P < 0.01$) < *I. galbana* ($15.4 \pm 0.9 \times 10^6$) < *N. gaditana* ($47.7 \pm 2.0 \times 10^6$, $P < 0.001$). On the other hand, the cellular dry weight (pg $cell^{-1}$) of each species increased in the inverse sense of steady state densities: *N. gaditana* (8.0 ± 0.3) < *I. galbana* (22.3 ± 2.8) < *R. lens* (120.8 ± 6.4) < *T. suecica* (220.7 ± 11.5, $P < 0.001$). Regarding the daily productivity, defined as the dry weight (DW) of harvested culture per liter of culture per day (mg l^{-1} day^{-1}), similar values were found in *R. lens* and *T. suecica* cultures (133 ± 5 and 129 ± 9 mg l^{-1} day^{-1}, respectively), being lower in *N. gaditana* and *I. galbana* cultures (116 ± 4 and 103 ± 6 mg l^{-1} day^{-1}, respectively).

The gross biochemical composition (% DW) of the microalgal species is shown in Fig. 1. *R. lens* showed a higher protein content (55%) than the remaining species ($P < 0.05$), whereas maximum lipid content was found in both *I. galbana* and *N. gaditana* (25–27%, $P < 0.001$). Maximum carbohydrate was found in *T. suecica* (16%, $P < 0.01$), followed by *R. lens* (11%) and by *I. galbana* and *N. gaditana* (both with 9%). Significant differences in the C:N ratio were also found among species (Fig. 1), with *R. lens* showing the lowest value (4.5 ± 0.2), followed by *T. suecica* (5.2 ± 0.2), *N. gaditana* (7.5 ± 0.01) and *I. galbana* (8.8 ± 0.2, $P < 0.05$). These results supported the gross composition found for the different microalgal species. *R. lens* contained 7.8 ± 0.3 pg $cell^{-1}$ of the pigment phycoerythrin, which corresponded to circa 12% of its total protein content.

Maximal productivity of protein per day was achieved in *R. lens* culture (72.2 mg protein l^{-1} day^{-1}), followed by *T. suecica* (57.5 mgprotein l^{-1} day^{-1}), while lower values were found in *I. galbana* and *N. gaditana* cultures (45.8 and 47.6 protein l^{-1} day^{-1}, respectively).

In the second experiment the steady state densities found for both *R. lens* and *T. suecica* cultures and their gross biochemical composition were highly reproducible and very similar to values found in the first experiment.

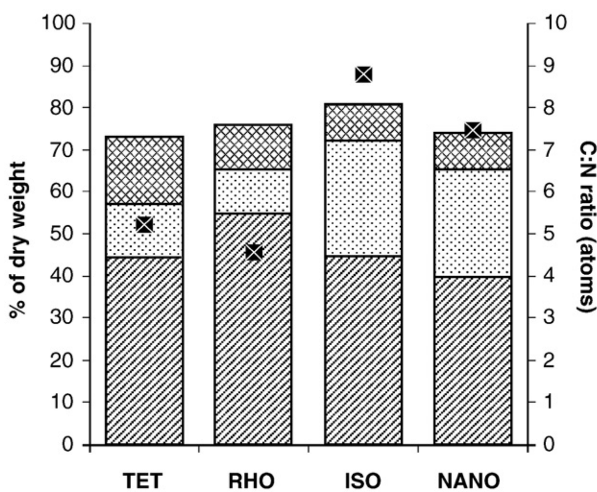

Fig. 1. Gross biochemical composition (% of dry weight) and C:N ratio (atoms) of the different microalgal species cultured semi-continuously in nutrient saturated conditions and with a daily renewal rate of 30%. TET (*Tetraselmis suecica*), RHO (*Rhodomonas lens*), ISO (*Isochrysis galbana*), NANO (*Nannochloropsis gaditana*). ▨ Protein, ▤ Lipid, ▩ Carbohydrate, ☒ C:N ratio. Data are mean values ($n = 3$, cultures harvested in three different days). Standard deviations were all below 10% of mean values and are not shown.

3.2. Fatty acid and amino acid composition of T. suecica and R. lens

Important differences in the total fatty acid (FA) composition were found between *T. suecica* and *R. lens* (Table 2). The major FA found in *T. suecica* was the saturated palmitic acid 16:0 (37%), whereas in *R. lens* it was the polyunsaturated linolenic acid 18:3$n-3$ (26% of total FA). The sum of saturated FA was nearly 30% in *R. lens* and 44% in *T. suecica*, whereas monoenes represented 7 and 16%, respectively (Table 2). Polyunsaturated fatty acids (PUFAs) were clearly higher in *R. lens* (circa 61%) than in *T. suecica* (40%). The levels of eicosapentaenoic acid (EPA, 20:5$n-3$) were higher in *R. lens* (8.4%) than in *T. suecica* (4.7%), while docosahexaenoic acid (DHA, 22:6$n-3$) was only found in *R. lens* (6.9%).

Regarding the total amino acid (AA) composition of *T. suecica* and *R. lens* (Table 3), major differences between species were more quantitative than qualitative. The amount of AA per single cell was 49.2 pg $cell^{-1}$ in *R. lens*, which corresponded to nearly 40% of its DW, whereas *T. suecica* contained 42.5 pg AA $cell^{-1}$, representing almost 20% of its DW.

3.3. Growth and survival of Artemia sp.

Survival of *Artemia* at the end of 8 days was higher in both groups ARHO and ATET (88 ± 4% and 83 ± 8%, respectively), than in AISO (69 ± 4%, $P < 0.01$), or than in ANANO (18 ± 3%, $P < 0.001$). However, the mortality of *Artemia* individuals in groups ARHO, ATET and AISO ocurred mainly in the first two days of the experiment (data not shown), as survival remained stable until the end of the experiment. In contrast, the mortality of individuals in ANANO was gradual along the experiment (in the second day survival was 81 ± 8%). Growth of individuals was markedly faster in group ARHO than in the remaining groups (Fig. 2). At day 8, the length of *Artemia* from ARHO (4.9 ± 0.6 mm) was considerably higher than in the remaining groups ($P < 0.001$). The same trend was observed for the DW of individuals, which decreased in the sense: ARHO (111.2 ± 8.6 µg $Artemia^{-1}$) > ATET (96.6 ± 5.2 µg $Artemia^{-1}$) > AISO (81.3 ± 7.9 µg $Artemia^{-1}$) > ANANO (25.7 ± 3.6 µg $Artemia^{-1}$) ($P < 0.001$).

In the second experiment survival of individuals from groups ARHO and ATET was in the same range as in the first experiment, and again no significant difference between groups were found ($P > 0.05$). Differences in *Artemia* length could be already observed from day 2 onward (Fig. 3). At the end of 5 days, juveniles from ARHO had a higher length (3.6 ± 0.3 mm) than those from ATET (3.2 ± 0.4 mm, $P < 0.001$). Differences in the DW of 5-day old *Artemia* were also observed (Table 4), with juveniles from ARHO weighing more than those from ATET ($P < 0.01$). Feed conversion rate (FCR) was slightly higher in group ARHO than in ATET, being 6.5 and 6.2, respectively.

3.4. Biochemical composition of Artemia juveniles fed R. lens or T. suecica

Differences in the biochemical composition of *Artemia* juveniles from groups ATET and ARHO (Table 4) were smaller than in the supplied microalgae. No significant differences were found in protein and carbohydrate content, whereas lipid content was higher in juveniles from ATET (15.2%, $P < 0.01$). The inorganic fraction, calculated as the difference between the total dry weight and the organic fraction (protein + lipid + carbohydrate), was nearly the same in both groups (Table 4). The ingestion of the different microalgal species modulated to a certain extent the FA composition of *Artemia* juveniles (Table 2). The sum of saturated FA was nearly the same in both groups (35 to 38%), as well as the proportion of monoenes (27–28%) and PUFAs (both with 35%). The saturated palmitic acid (16:0) was the major FA found in both groups. Only juveniles from group ATET contained 16:4$n-3$ (3.8%), while DHA was only found in ARHO (1.1%). Arachidonic acid (20:4$n-6$) was found in very small amounts in both groups (≤0.4%), whereas EPA percentage was higher in group ARHO (6.2%) than in ATET (4.2%, $P < 0.05$).

Table 2
Fatty acid (FA) composition (% of total FA) of *Tetraselmis suecica* and *Rhodomonas lens* cultured semi-continuously with a daily renewal rate of 30% of the volume of cultures and of 5-day old *Artemia* fed those microalgal species.

Fatty acid	Microalgal species		5-day old *Artemia*	
	T. suecica	*R. lens*	ATET	ARHO
14:0	6.5 ± 0.2	7.6 ± 0.7	0.7 ± 0.1	1.3 ± 0.6
16:0	37.1 ± 2.4	21.2 ± 1.0	29.8 ± 2.1	24.2 ± 1.3
16:1*n* − 9	1.5 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	1.2 ± 0.3
16:1*n* − 7	2.2 ± 0.1	2.6 ± 0.7	0.3 ± 0.1	1.4 ± 0.6
16:4*n* − 3	14.6 ± 1.1	n.f.	3.8 ± 0.6	n.f.
18:0	0.0	0.8 ± 0.0	4.7 ± 1.2	12.0 ± 0.9
18:1*n* − 9	9.9 ± 0.9	0.7 ± 0.0	20.1 ± 1.2	3.3 ± 1.1
18:1*n* − 7	1.1 ± 0.1	2.8 ± 0.4	6.0 ± 0.6	20.9 ± 1.8
18:2*n* − 6	1.4 ± 0.1	0.4 ± 0.1	1.9 ± 0.1	0.3 ± 0.1
18:3*n* − 3	11.8 ± 1.4	26.4 ± 1.5	15.8 ± 0.7	20.5 ± 1.3
18:4*n* − 3	7.3 ± 0.8	18.3 ± 1.5	9.8 ± 0.9	6.0 ± 1.5
20:1*n* − 9	1.2 ± 0.1	n.f.	0.4 ± 0.1	0.1 ± 0.1
20:4*n* − 6	0.2 ± 0.0	0.0	0.1 ± 0.0	0.4 ± 0.1
20:4*n* − 3	0.4 ± 0.1	1.1 ± 0.2	0.4 ± 0.1	0.7 ± 0.1
20:5*n* − 3	4.7 ± 0.8	8.4 ± 0.6	4.1 ± 0.6	6.2 ± 0.5
22:6*n* − 3	n.f.	6.9 ± 0.7	n.f.	1.1 ± 0.2
Others	0.1	1.7	0.8	0.4
Saturated	43.6 ± 2.4	29.6 ± 0.5	35.7 ± 0.9	37.5 ± 0.7
Monoenes	15.9 ± 1.0	7.2 ± 1.2	28.0 ± 2.0	26.8 ± 1.3
PUFAs	40.5 ± 3.2	61.4 ± 1.2	36.1 ± 1.7	35.2 ± 2.0
n − 3	38.9 ± 3.3	61.0 ± 1.3	34.2 ± 1.7	34.5 ± 1.8
n − 6	1.6 ± 0.1	0.4 ± 0.1	2.0 ± 0.1	0.7 ± 0.1
DHA/EPA	–	0.8	–	0.2

ATET: *Artemia* fed *T. suecica*; ARHO: *Artemia* fed *R. lens*. PUFAs: polyunsaturated fatty acids. Values are means ± S.D. ($n = 3$). Data of microalgae refers to cultures harvested in three different days. n.f.: not found. Values shown as 0.0 are below 0.05. Different superscript letters indicate significant differences between groups ($\alpha = 0.05$).

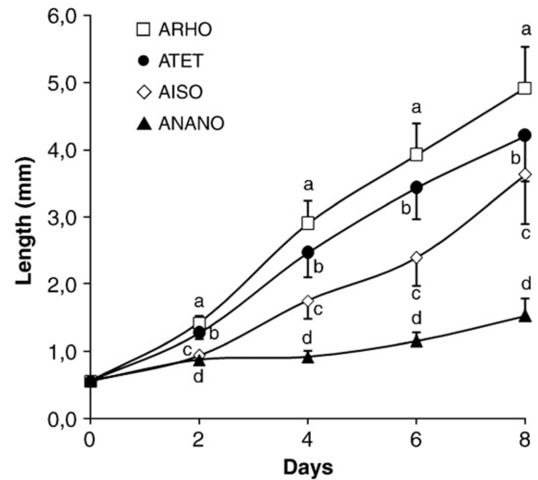

Fig. 2. Growth of *Artemia* fed on four different microalgal species for 8 days. ARHO: group fed *Rhodomonas lens*; ATET: *Tetraselmis suecica*; AISO: *Isochrysis galbana*; ANANO: *Nannochloropsis gaditana*. Data are means ± S.D. ($n = 3$, 25 individuals measured per replicate). Different superscript letters within the same day indicate significant differences among groups ($P < 0.001$).

4. Discussion

4.1. Microalgae cultures

Although other authors pointed out the instability and unpredictable growth of *Rhodomonas* sp. in batch cultures, and suggested a 30% exchange every three days as convenient to maintain cultures in exponential growth (Knuckey et al., 2005), no problem was encountered for the establishment of steady semi-continuous cultures of *R. lens* with a daily renewal rate of 30% of the total volume, being highly reproducible and stable along time. The better results obtained in the present work may be due to the high N concentration used (4 mM N l^{-1}), in comparison to the *f* medium used by other authors (Knuckey et al., 2005). In fact, the steady state density attained in this study for *R. lens* (3.7×10^6 cells ml^{-1}) was 2 to 10-fold higher than densities reported by other authors for *Rhodomonas* sp. in log, late-log or stationary phase using lower nutrient concentrations (Renaud et al., 1999, 2002; Dunstan et al., 2005; Lafagarta-De La Cruz et al., 2006). Nevertheless, it should be emphasized that previous experiments of *R. lens* culture carried out in our laboratory also showed this species to be more sensitive than other microalgae usually used in aquaculture, being more sensitive to high light conditions and dying faster than other species once nitrogen is depleted from culture media (Coutinho, 2008).

The productivity of the different microalgal species cultured semi-continuously in 5 l glass flasks was in general lower than values reported previously when using 30 mm diameter glass tubes containing 80 ml of culture volume, with the same nutrient concentration and daily renewal rate conditions (Otero and Fábregas, 1997; Otero et al., 1997; Coutinho, 2008; Ferreira et al., 2009). These differences could be explained by the lower effective irradiance to cells in 5 l glass flasks in comparison with thin glass tubes, and confirms that light was the limiting factor of algal growth in the system, as indicated by the presence of nitrate in the harvested cultures. Results demonstrate the importance of the use of efficient culture systems that maximize irradiance availability.

The daily productivity of *R. lens* (DW l^{-1} day^{-1}) was 15 to 30% higher than *N. gaditana* and *I. galbana* cultures, respectively, but equal to *T. suecica* culture. However, the protein productivity was by far much higher in *R. lens* culture (25 to 55% higher than the remaining

Table 3
Total amino acid composition (in pg $cell^{-1}$ and in % of total amino acid) of *Tetraselmis suecica* and *Rhodomonas lens* cultured semi-continuously with a daily renewal rate of 30% of the volume of cultures.

EAA	*T. suecica*		*R. lens*		NEAA	*T. suecica*		*R. lens*	
	pg $cell^{-1}$	%	pg $cell^{-1}$	%		pg $cell^{-1}$	%	pg $cell^{-1}$	%
Arginine	3.4	8.0	4.0	8.2	Alanine	2.3	5.3	3.9	7.9
Histidine	1.4	3.3	1.1	2.2	Asp+Asn	2.5	5.8	5.3	10.7
Isoleucine	1.9	4.4	2.4	4.8	Cystine	0.04	0.1	0.3	0.6
Leucine	4.0	9.5	4.2	8.5	Glu+Gln	3.5	8.3	6.8	13.9
Valine	2.6	6.2	3.2	6.5	Glycine	3.7	8.7	3.2	6.5
Lysine	1.0	2.4	2.7	5.5	Proline	2.2	5.2	2.0	4.0
Phenylalanine	4.3	10.2	2.7	5.5	Serine	1.9	4.4	2.0	4.0
Methionine	1.5	3.4	1.4	2.9	Tyrosine	2.9	6.8	2.1	4.3
Threonine	3.3	7.8	1.9	3.9					
Total	23.5	55.3	23.6	48.0	Total	19.0	44.7	25.5	52.0

EAA: essential amino acids, NEAA: non essential amino acids. Values from a single analysis.
Asp+Asn: aspartic acid plus asparagine; Glu+Gln: glutamic acid plus glutamine.

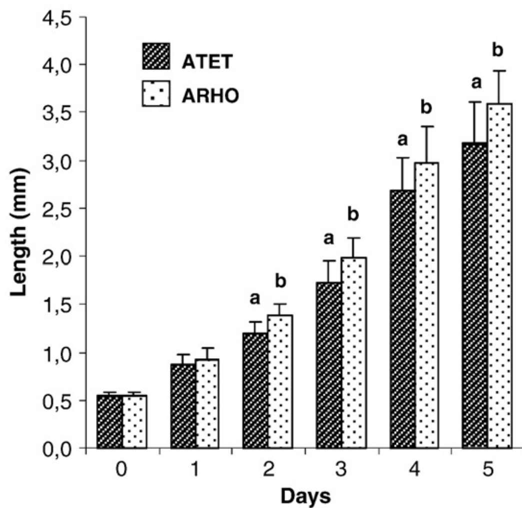

Fig. 3. Growth of *Artemia* fed either *Tetraselmis suecica* (ATET) or *Rhodomonas lens* (ARHO) for 5 days (experiment 2). Data are means ± S.D. ($n=3$, 25 individuals measured per replicate). Different superscript letters within the same day indicate significant differences between groups ($P<0.001$).

cultures). However, this high protein content was achieved at the expense of using twice nutrient concentration than in *I. galbana* or *N. gaditana* cultures, in order to obtain N saturation conditions. Still, a concentration of 4 mM N l^{-1} was also in excess for the conditions selected (results not shown), and probably the same result could have been obtained with a nutrient concentration slightly lower. It should be kept in mind that it is desirable to adjust nutrient concentration at the exact point in which nutrients turn from saturation to limitation conditions, not only to optimize cost productions but also to avoid the possible negative effect of excess nitrate (Fábregas et al., 1998). The high protein content of *R. lens* can be partially explained by the accumulation of phycoerithrin (7.8 ± 0.3 pg $cell^{-1}$) that represented 12% of total protein. Phycoerythrin is a protein located inside the thylakoids of *R. lens* (Ludwig and Gibbs, 1989) that functions as light-harvesting and energy-transfer pigment in photosynthesis. Moreover, phycoerythrin production is favoured by the light limited conditions produced in the glass flask system, since lower levels of this pigment are achieved in 30 mm glass tubes (Coutinho, 2008). Other authors reported maximum values of phycoerythrin of 4.9 pg $cell^{-1}$ in dense cultures of *Rhodomonas salina*, cultured under different irradiance levels but using *f*/2 medium as nutrient concentration (Bartual et al., 2002). Phycoerythrin content seems to be strongly dependent on effective light intensity and nutrient availability.

Table 4
Dry weight, feed conversion rate (FCR), biochemical composition (% of dry weight) and caloric value (in J *Artemia*$^{-1}$) of 5-day old *Artemia* fed *T. suecica* (ATET) or *R. lens* (ARHO).

	ATET	ARHO
Dry weight (µg *Artemia*$^{-1}$)	50.9 ± 4.3[b]	60.5 ± 3.3[a]
Protein (%)	63.6 ± 2.6	67.7 ± 1.2
Lipid (%)	15.2 ± 0.6[a]	12.3 ± 0.6[b]
Carbohydrate (%)	10.0 ± 1.1	8.3 ± 0.4
Inorganic fraction (%)	11.2 ± 1.4	11.7 ± 1.4
Caloric value	1.16 ± 0.05[b]	1.35 ± 0.02[a]
FCR	6.2	6.5

Inorganic fraction calculated as 100% − ΣOrganic fraction. FCR = total supplied food (dry weight)/biomass of *Artemia* attained (dry weight). Data are means ± S.D., $n=3$, except for dry weight ($n=5$). Different superscript letters indicate significant differences between groups ($P<0.01$).

Regarding the composition of microalgae, the protein content of *T. suecica* (45% of DW) found in this study was considerably higher than values reported by other authors (16 to 31%) for the same species cultured with different nutrient mediums (Brown, 1991; D'Souza and Kelly, 2000), but was similar to values described by other authors when using similar media formulations and culture conditions (Otero and Fábregas, 1997; Seixas et al., 2008). *R. lens* was shown to contain circa 55% protein, which is higher or similar to percentages found by other authors for *Rhodomonas salina* (48 to 59%, Brown et al., 1998; McCausland et al., 1999) or for *Rhodomonas* sp. (Renaud et al., 1999; Renaud et al., 2002; Dunstan et al. 2005). Results demonstrate the effectiveness of using daily renewal rates and nutrient saturation conditions to reach maximal protein content in the microalgae, as well as to obtain daily biomass of stable biochemical composition that was highly reproducible in the second experiment.

The C:N ratios found in *R. lens* and *T. suecica* cultures were very similar to values previously described by Seixas et al. (2008) for the same microalgae (4.4 and 5.2, respectively), when cultured in 1 l glass flasks in nutrient saturated conditions and with the same daily dilution rate. These values were lower than the predicted by the Redfield ratio (C:N = 6.6) for microalgae under non-limited nutrient conditions. However, this same observation had been previously reported by Otero et al. (1998) in *Phaeodactylum tricornutum* cultures maintained under light–dark photoperiods and in nutrient saturated conditions, who suggested that carbon respiratory losses during the dark period could explain these lower C:N ratios observed when harvesting the microalgae at the beginning of the light period. As for *N. gaditana*, the C:N ratio found in this study (7.5) was similar to the values reported by Ferreira et al. (2009) for *N. gaditana* cultured in glass tubes with 30 mm diameter (80 ml volume) under nutrient saturated conditions. Regarding *I. galbana* culture, the C:N ratio that was found in this work (8.8) was also similar to values reported by Seixas et al. (2008) for the same species cultured in 1 l glass flasks (8.0), or than Ferreira et al. (2008) for *I. galbana* T-ISO (8.0) cultured in 80 ml glass tubes. The slightly higher C:N values reported in the present work could be partially attributed to the accumulation of pigment and lipid in response to the lower light availability in the 5 l glass flasks. The lower C:N ratios found in both *R. lens* and *T. suecica* in comparison with *I. galbana* and *N. gaditana*, could be partially explained by the considerably higher lipid levels found *I. galbana* and *N. gaditana*. This could be due to intrinsic characteristics of each microalgal species, having higher or lower tendency for lipid accumulation, or to the relation volume:surface of cells. Bigger cells such as *T. suecica* and *R. lens* have a lower surface of cell membrane in relation to its volume, and as a consequence lower lipid percentages (of its dry weight), that could affect the C:N ratio.

Regarding the total amino acid (AA) composition of *R. lens* and *T. suecica*, values found for *T. suecica* were similar to those reported by Brown (1991) except for arginine and lysine, which were lower in the present work, 8 and 2.4%, respectively, compared to 13.2 and 6.0% reported by that author. The percentages of total AA found in *R. lens* were also closely related to those described by Dunstan et al. (2005) for *Rhodomonas* sp. The amount of intracellular AA in microalgae increases strikingly with increasing growth rate in cultures which are not deprived of N (Flynn, 1990). When analysing the AA content of about 40 species of microalgae from seven algal classes, Brown et al. (1997) found that they were all very similar in composition (weight % of total AA), with few exceptions being observed (as the very high content of arginine in *Tetraselmis* sp.). However, in the present work *R. lens* was found to contain much higher amounts of total AA (41% of DW) in comparison with *T. suecica* (19%). This difference is considerably higher than the difference revealed by protein analysis using the Lowry method (55% and 45% of DW respectively). The lower amount of total AA in both microalgal species, compared to total protein, is probably the result of missing values of AA which were not quantified (e.g. tryptophan, and other free AA such as taurine, hydroxiproline, etc.) or to the possible overestimation of protein by the

Folin-phenol method, as previously described for other microalgae (Berges et al., 1993). The differences in the AA content between both species may be a result from the intrinsic presence of phycoerythrin in *R. lens* and to and hypothetical accumulation of higher amounts of intracellular free amino acids to build up this pigment.

As for the FA composition of *R. lens*, the percentages of EPA and DHA found in the present work (8.4% and 6.9% of total FA, respectively) were very similar to values reported by other authors for *Rhodomonas* sp. (Renaud et al., 1999; Dunstan et al., 2005). These authors also reported the presence of high levels of the polyunsaturated FA 18:3$n-3$ and 18:4$n-3$, as found for *R. lens* in this study (26.4 and 18.3%, respectively).

4.2. Artemia growth and biochemical composition

The establishment of comparisons of *Artemia* growth among different works is always difficult due to differences in many rearing parameters, such as temperature, *Artemia* strains, handling conditions, food diets, etc. Even when the same microalgal species is used, culture conditions strongly affect the nutritional value of microalgae to be harvested (Otero and Fábregas, 1997; D'Souza and Kelly, 2000) and therefore the use of continuous or semi-continuous cultures is advisable.

After examining the data reported by several authors (Table 1) about *Artemia* rearing conditions and microalgal diets, and taking also into consideration the present results, it seems reasonable to state that feeding *Artemia* with *R. lens* results in faster growth of individuals than with any other microalgal diets previously reported in the literature, only comparable to other cryptophytes such as *Cryptomonas* sp. (Thinh et al., 1999). Our results also corroborate previous observations found for the optimal development of the calanoid *Acartia sinjiensis* fed on *Rhodomonas* sp. (Knuckey et al., 2005). These findings lead to the assumption that Cryptophytes may in fact represent an excellent diet to improve the growth of live prey and other filter-feeders. Previous works with filtering molluscs have shown that *Rhodomonas salina* constitutes a high-quality food for Pacific oyster *Crassostrea gigas* spat (Brown et al., 1998), and improves metamorphosis rate and culture productivity of *Pecten maximus* when used as a supplement of other "standard" microalgae used in hatcheries (Tremblay et al., 2007).

Another interesting feature concerning *Artemia* growth is related with the size of microalgal species. When analysing our results a certain tendency for *Artemia* to grow better with "large size" microalgae can be observed. Taking as examples the sizes described by Brown et al. (1997) for *Tetraselmis* sp. (15 × 9 µm), *Rhodomonas* sp. (10 × 12 µm), *Dunaliella tertiolecta* (10 × 12 µm), *I. galbana* (3 × 5 µm) and *Nannochloropsis* sp. (3 µm), we can observe that larger microalgae gave, in general, better results of *Artemia* growth even when the protein content is similar between species (e.g. *T. suecica* and *I. galbana*). Even if *Artemia* can filter particles ranging in size from 1 to 50 µm (D'Agostino, 1980; Van Stappen, 1996), other studies have shown that ranges between 7 and 28 µm are preferable and an optimum size of 16.0 µm has been pointed out (Fernández, 2001). In the case of *N. gaditana*, the low digestibility of their cell walls, besides its small size, could explain the poor performance using this species, as the gross biochemical composition of *N. gaditana* was similar to *I. galbana*, and this last one generated better growth of *Artemia*. Indeed, not all microalgal species are suitable to feed *Artemia* as previously described (Sick, 1976; reviewed by Dhont and Lavens, 1996). For example, *Chlorella* and *Stichococcus* have a thick cell wall that cannot be digested by *Artemia* and the genera *Coccochloris* produce gelatinous substances that interfere with food uptake during the filtering process.

Another interesting feature that we observed in this work and in previous experiments, is that the growth of *Artemia* with *Isochrysis* sp. can be as fast as with other "good" microalgal diets as soon as *Artemia* have attained a size of about 2.0–2.5 mm (Seixas et al., 2008). Growth rate of *Artemia* with *I. galbana* between days 6 and 8 was even higher than with *R. lens* (see growth rate in Fig. 2). Evjemo and Olsen (1999) have also shown a slow development of *A. franciscana* fed *I. galbana* T-ISO in the first days of development, and a considerable increase in the growth rate of juveniles after they reached a size of 2.4 mm. This result may indicate a change in the nutritional requirements of the *Artemia*, or may be the result of changes in the digestive capacity of *Artemia* in later development stages than metanauplii, that may deserve further investigation.

Differences in the biochemical composition of *Artemia* juveniles fed *R. lens* or *T. suecica* were also found, especially in their fatty acid profile. Juveniles fed *R. lens* contained 50% more EPA than those fed *T. suecica* and a small percentage of DHA (1.1%), reflecting the ingested diet. Similar percentages of DHA were also found in juvenile *Artemia* enriched with *I. galbana* T-ISO (1.2% in 1.5 mm *Artemia* and 3.4% in 2.5 mm *Artemia*) or with *C. muelleri* (≤0.8%) (Ritar et al., 2003, 2004). It is well known that HUFAs are essential in diets for crustacean and other marine larvae (Sorgeloos et al., 1998; Bell et al., 2003), and as for nauplii, the lipid composition of *Artemia* biomass might be modulated by the supplied diet, with the additional advantage of having a higher filtration capacity (Dhont and Van Stappen, 2003). Regarding the gross biochemical composition of Artemia juveniles, their protein content (64–68%) was within the highest values reported for adult *Artemia* sp. (39 to 67%) (Dhont and Van Stappen, 2003), whereas lipid and carbohydrate content (4–31% and 4–20%, respectively) were in the normal ranges pointed out by those authors.

Further studies of the total and free amino acid composition of *Artemia* nauplii/juveniles and other live prey such as the rotifer *Brachionus plicatilis* enriched with *R. lens* would be useful to confirm the high nutritional value of this microalgal diet. Helland et al. (2000) showed that the free AA pool of *Artemia franciscana* nauplii was considerably higher in nauplii enriched for 12 h or 24 h with *T. suecica* or *C. gracilis* than in unenriched nauplii, especially in essential AA and in taurine.

In conclusion, the Cryptophyte *R. lens* was confirmed as an excellent monodiet to optimize the growth of *Artemia* sp. and to improve the nutritional composition of *Artemia* juveniles. However, as shown for the copepod *Acartia tonsa* when fed mixed algal diets of diatoms and dinoflagellates (Jones and Flynn, 2005), further studies using combinations of *R. lens* and other microalgae would be useful to test further improvements of growth and of the nutritional composition of different stages of *Artemia*.

Acknowledgments

Pedro F. Seixas was supported by a PhD grant in the period 2004–2008 (Ref. SFRH/BD/16419/2004) by FCT (Fundação para a Ciência e a Tecnologia), Portugal. **[SS]**

References

Abreu-Grobois, F.A., Briseño-Dueñas, R., Herrera, M.A., Malagón, M.L., 1991. A model for growth of *Artemia franciscana* cultures based on food ration-dependent gross growth efficiencies. Hydrobiologia 212, 27–37.

Aragão, C., Conceição, L., Dinis, M., Fyhn, H.J., 2004. Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. Aquaculture 234, 429–445.

Bartual, A., Lubián, L.M., Gálvez, J.A., Niell, F.X., 2002. Effect of irradiance on growth, photosynthesis, pigment content and nutrient consumption in dense cultures of *Rhodomonas salina* (Wislouch) (Cryptophyceae). Cienc. Mar. 28, 381–392.

Bell, J.G., McEvoy, L.A., Estevez, A., Shields, R.J., Sargent, J.R., 2003. Optimising lipid nutrition in first-feeding flatfish larvae. Aquaculture 227, 211–220.

Bennet, A., Bogorad, L., 1973. Complementary chromatic adaptation in a filamentous blue-green alga. J. Cell Biol. 58, 419–435.

Berges, J.A., Fisher, A.E., Harrison, P.J., 1993. A comparison of Lowry, Bradford, and Smith protein assays using different protein standards and protein purified from the marine diatom *Thalassiosira pseudonana*. Mar. Biol. 115, 187–193.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Brown, M.R., 1991. The amino-acid and sugar composition of 16 species of microalgae used in mariculture. J. Exp. Mar. Biol. Ecol. 145, 79–99.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., Dunstan, G.A., 1997. Nutritional properties of microalgae for mariculture. Aquaculture 234, 315–331.
Brown, M.R., McCausland, M.A., Kowalski, K., 1998. The nutritional value of four Australian microalgal strains fed to Pacific oyster *Crassostrea gigas* spat. Aquaculture 165, 281–293.
Bryant, D.A., Guglielmi, G., Marsac, N.T., Castets, A.M., Cohen-Bazire, G., 1979. The structure of cyanobacterial phycobilisomes: a model. Arch. Microbiol. 123, 113–127.
Clesceri, L.S., Greenberg, A.E., Trussell, R.R., 1989. (Eds.) Standard methods for the examination of water and wastewater. American Public Health Association, American Waterworks Association, Water Pollution Control Federation. Washington D.C., 2134 pp.
Conklin, D.E., 1995. Digestive physiology and nutrition. In: Factor, J.R. (Ed.), Biology of the Lobster *Homarus americanus*. Academic Press, San Diego, California, pp. 441–458.
Coutinho, P., 2008. La microalga marina *Rhodomonas lens*: optimización de las condiciones de cultivo y potencial biotecnológico. PhD Thesis. University of Santiago de Compostela, Spain. Pp. 261.
D'Agostino, A.S., 1980. The vital requirements of *Artemia*: physiology and nutrition. In: Personne, G., Sorgeloos, P., Roels, O., Jaspers, E. (Eds.), The Brine Shrimp *Artemia*. Physiology, Biodiversity, Molecular & Biology, vol. 2. Universa Press, Wetteren, Belgium, pp. 55–82.
Dhert, P.H., Bombeo, R.B., Sorgeloos, P., 1993. Use of ongrown *Artemia* in nursery culturing of the tiger shrimp. Aquacult. Int. 1, 170–177.
Dhert, P.H., Rombaut, G., Suantika, G., Sorgeloos, P., 2001. Advancement of rotifer culture and manipulation techniques in Europe. Aquaculture 200, 129–146.
Dhont, J., Lavens, P., 1996. Tank production and use of ongrown *Artemia*. In: Sorgeloos, P., Lavens, P. (Eds.), Manual on the Production and Use of Live Food for Aquaculture. Fisheries Technical Paper No. 361. Food and Agriculture Organization of the United Nations, Rome, pp. 164–194.
Dhont, J., Van Stappen, G., 2003. Biology, tank production and nutritional value of *Artemia*. In: Støttrup, J.G., McEvoy, L.A. (Eds.), Live Feeds in Marine Aquaculture. Blackwell Science Ltd, pp. 145–195.
Domingues, P.M., Sykes, A., Andrade, J.P., 2001. The use of *Artemia* sp. or mysids as food source for hatchlings of the cuttlefish (*Sepia officinalis* L.); effects on growth and survival throughout the life cycle. Aquac. Int. 9, 319–331.
D'Souza, F.L., Kelly, G.J., 2000. Effects of a diet of a nitrogen-limited alga (*Tetraselmis suecica*) on growth, survival and biochemical composition of tiger prawn (*Penaeus semisulcatus*) larvae. Aquaculture 181, 311–329.
Dunstan, G.A., Brown, M.R., Volkman, J.K., 2005. Cryptophyceae and rhodophyceae; chemotaxonomy, phylogeny, and application. Phytochemistry 66, 2557–2570.
Enright, C.T., Newkirk, G.F., Craigie, J.S., Castell, J.B., 1986. Evaluation of phytoplankton as diets for juvenile *Ostrea edulis* L. J. Exp. Mar. Biol. Ecol. 96, 1–13.
Evjemo, J.O., Olsen, Y., 1999. Effect of food concentration on the growth and production rate of *Artemia franciscana* feeding on algae (T.iso). J. Exp. Mar. Biol. Ecol. 242, 273–296.
Fábregas, J., Abalde, J., Herrero, C., Cabezas, B., Veiga, M., 1984. Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. Aquaculture 42, 207–215.
Fábregas, J., Otero, A., Morales, E., Cordero, B., Patiño, M., 1996. *Tetraselmis suecica* cultured in different nutrient concentrations varies in nutritional value to *Artemia*. Aquaculture 143, 197–204.
Fábregas, J., Otero, A., Morales, E.D., Arredondo-Vega, B.O., Patiño, M., 1998. Modification of the nutritive value of *Phaeodactylum tricornutum* for *Artemia* sp. in semicontinuous cultures. Aquaculture 169, 167–176.
Fábregas, J., Otero, A., Dominguez, A., Patiño, M., 2001. Growth rate of the microalga *Tetraselmis suecica* changes the biochemical composition of *Artemia* species. Mar. Biotechnol. 3, 256–263.
Fernández, R.G., 2001. *Artemia* bioencapsulation I. Effect of particle sizes on the filtering behavior of *Artemia franciscana*. J. Crust. Biol. 21, 435–442.
Ferreira, M., Maseda, A., Fábregas, J., Otero, A., 2008. Enriching rotifers with "premium" microalgae. *Isochrysis* aff. *galbana* clone T-ISO. Aquaculture 279, 126–130.
Ferreira, M., Coutinho, P., Seixas, P., Fábregas, J., Otero, A., 2009. Enriching rotifers with "premium" microalgae. *Nannochloropsis gaditana*. Mar. Biotechnol. 11, 585–595.
Flynn, K.J., 1990. Composition of intracellular and extracellular pools of amino acids, and amino acid utilization of microalgae of different sizes. J. Exp. Mar. Biol. Ecol. 139, 151–166.
Flynn, K.J., Davidson, K., Cunningham, A., 1996. Prey selection and rejection by a microflagellate; implications for the study and operation of microbial food webs. J. Exp. Mar. Biol. Ecol. 196, 357–372.
Gamallo, Y., 1992. Cultivo de *Artemia* sp. con microalgas marinas: crecimiento, supervivencia, reproducción y composición bioquímica. PhD Thesis. University of Santiago de Compostela, Spain. 180 pp. (In Spanish).
García-Ulloa, G.M., Gamboa, J., Zavala, J.L., Ogura, T., Lavens, P., 1999. Influence of different diets on length and biomass production of brine shrimp *Artemia franciscana* (Kellog, 1906). Bul. Mar. Coast. Res. 28, 7–18.
Ginzburg, M., 1987. *Dunaliella*: a green alga adapted to salt. Adv. Bot. Res. 14, 93–183.
Godínez, D.E., Carmen Gallo, M., Gelabert, R., Díaz, A., Gamboa, J., Landa, V., Godínez, R., 2004. Crecimiento larvario de *Artemia franciscana* (Kellog, 1906) alimentada con dos especies de microalgas vivas. Zootec. Trop. 22, 265–275 (in Spanish, with English abstract).
Helland, S., Triantaphyllidis, G.V., Fyhn, H.J., Evjen, M.S., Lavens, P., Sorgeloos, P., 2000. Modulation of the free amino acid pool and protein content in populations of the brine shrimp *Artemia* spp. Mar. Biol. 137, 1005–1016.
Iglesias, J., Sánchez, J., Bersanob, J., Carrasco, J., Dhont, J., Fuentes, L., Linares, F., Muñoz, J., Okumura, S., Roo, J., van der Meeren, T., Vidal, E., Villanueva, R., 2007. Rearing of *Octopus vulgaris* paralarvae: present status, bottlenecks and trends. Aquaculture 266, 1–15.
Jones, R.H., Flynn, K.J., 2005. Nutritional status and diet composition affect the value of diatoms as copepod prey. Science 307, 1457–1459.
Knuckey, R.M., Semmens, G.L., Mayer, R.J., Rimmer, M.A., 2005. Development of an optimal microalgal diet for the culture of the calanoid copepod *Acartia sinjiensis*: effect of algal species and feed concentration on copepod development. Aquaculture 249, 339–351.
Kochert, G., 1978. Carbohydrate determination by the phenol–sulfuric acid method. In: Hellebust, J.A, Craigie, J.S. (Eds.), Handbook of Phycology Methods: Physiological and Biochemical methods. Cambridge University Press, London, pp. 95–97.
Koski, M., Breteler, W.K., Schogt, N., 1998. Effect of food quality on rate of growth and development of the pelagic copepod *Pseudocalanus elongatus* (Copepoda, Calanoida). Mar. Ecol. Prog. Ser. 170, 169–187.
Lafagarta-De La Cruz, F., Valenzuela-Espinosa, E., Millán-Núñez, R., Trees, C.C., Santamaría-del-Ángel, E., Nuñéz-Cebrero, F., 2006. Nutrient uptake, chlorophyll *a* and carbon fixation by *Rhodomonas* sp. (Cryptophyceae) cultured at different irradiance and nutrient concentrations. Aquacult. Eng. 35, 51–60.
Lim, L.C., Soh, A., Dhert, P., Sorgeloos, P., 2001. Production and application of on-grown *Artemia* in freshwater ornamental fish farm. Aquacult. Econ. Manage. 5, 211–228.
Lora-Vilchis, M.C., Cordero-Esquivel, B., Voltolina-Lobina, D., 2004. Growth of *Artemia franciscana* fed with *Isochrysis* sp. and *Chaetoceros muelleri* during its early life stages. Aquac. Res. 35, 1086–1091.
Lowry, O.H., Resobrough, H.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193, 265–275.
Ludwig, M., Gibbs, S., 1989. Localization of phycoerythrin at the luminal surface of the thylakoid membrane in *Rhodomonas lens*. J. Cell Biol. 108, 875–884.
McCausland, M.A., Brown, M.R., Barrett, S.M., Diemar, J.A., Heasman, M.P., 1999. Evaluation of live microalgae and microalgal pastes as supplementary food for juvenile Pacific oysters (*Crassostrea gigas*). Aquaculture 174, 323–342.
Marques, A., Dhont, J., Sorgeloos, P., Bossier, P., 2004. Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically grown brine shrimp *Artemia franciscana*. J. Exp. Mar. Biol. Ecol. 312, 115–136.
Marsh, J.B., Weinstein, D.B., 1966. Simple charring method for determination of lipids. J. Lipid Res. 7, 574–576.
Naegel, L., 1999. Controlled production of *Artemia* biomass using an inert commercial diet, compared with the microalgae *Chaetoceros*. Aquac. Eng. 21, 49–59.
National Research Council, 1993. Dietary requirements. Nutrient Requirements of Fish. National Academy Press, Washington D.C, pp. 3–33.
Otero, A., Fábregas, J., 1997. Changes in the nutrient composition of *Tetraselmis suecica* cultured semicontinuously with different nutrient concentrations and renewal rates. Aquaculture 159, 111–123.
Otero, A., García, D., Morales, E.D., Arán, J., Fábregas, J., 1997. Manipulation of the biochemical composition of the eicosapentaenoic acid-rich microalga *Isochrysis galbana* in semicontinuous cultures. Biotechnol. Appl. Biochem. 26, 171–177.
Otero, A., Domínguez, A., Lamela, T., García, D., Fábregas, J., 1998. Steady-states of semi-continuous cultures of a marine diatom: effect of saturating nutrient conditions. J. Exp. Mar. Biol. Ecol. 227, 23–34.
Otero, A., Patiño, M., Domínguez, A., Fábregas, J., 2002. Tailoring the nutritional composition of microalgae for aquaculture purposes — the use of semicontinuos culture techniques. Aquac. Eur. 33, 13–16.
Piña, P., Voltolina, D., Nieves, M., Robles, M., 2006. Survival, development and growth of the Pacific white shrimp *Litopenaeus vannamei* protozoea larvae, fed with monoalgal and mixed diets. Aquaculture 253, 523–530.
Reitan, K.J., Rainuzzo, J.R., Øie, G., Olsen, Y., 1997. A review of the nutritional effects of algae in marine fish larvae. Aquaculture 155, 207–221.
Renaud, S.M., Thinh, L., Parry, D.L., 1999. The gross chemical composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. Aquaculture 170, 147–159.
Renaud, S., Thinh, L., Lambrinidis, G., Parry, D., 2002. Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. Aquaculture 211, 195–214.
Ritar, A.J., Dunstan, G.A., Nelson, M.M., Brown, M.R., Nichols, P.D., Thomas, C.W., Smith, E.G., Crear, B.J., Kolkovski, S., 2004. Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation. Aquaculture 239, 351–373.
Ritar, A.J., Smith, G.G., Dunstan, G.A., Brown, M.R., Hart, P.R., 2003. *Artemia* prey size and mode of presentation: effects on survival and growth of phyllosoma larvae of southern rock lobster (*Jasus edwardsii*). Aquac. Int. 11, 163–182.
Sato, N., Murata, N., 1988. Membrane lipids. In: Packer, L., Glazer, A.N. (Eds.), Methods in Enzymology, Vol. 167. Academic Press, New York, pp. 251–259.
Scott, J.M., 1980. Effect of growth rate of the food alga on the growth/ingestion efficiency of a marine herbivore. J. Mar. Biol. Assoc. UK 60, 681–702.
Seixas, P., Rey-Méndez, M., Valente, L.M.P., Otero, A., 2008. Producing juvenile *Artemia* as prey for *Octopus vulgaris* paralarvae with different microalgal species of controlled biochemical composition. Aquaculture 283, 83–91.
Sick, L.V., 1976. Nutritional effect of five species of marine algae on the growth, development, and survival of the brine shrimp *Artemia salina*. Mar. Biol. 35, 69–78.
Sorgeloos, P., Lavens, P., Léger, P.H., Tackaert, W., Versichele, D., 1986. Manual for the Culture and Use of Brine Shrimp *Artemia* in Aquaculture. Artemia Reference Center, University of Ghent, Ghent, Belgium. (319 pp).
Sorgeloos, P., Coutteau, P., Dhert, P., Merchie, G., Lavens, P., 1998. Use of brine shrimp, *Artemia* spp., in larval crustacean nutrition: a review. Rev. Fish. Sci. 6, 55–68.
Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture 200, 147–159.
Støttrup, J.G., 2003. Production and nutritional value of copepods. In: Støttrup, J.G., McEvoy, L.A. (Eds.), Live Feeds in Marine Aquaculture. Blackwell Science Ltd, pp. 145–195.
Støttrup, J.G., Jensen, J., 1990. Influence of algal diet on feeding and egg-production of the calanoid copepod *Acartia tonsa* Dana. J. Exp. Mar. Biol. Ecol. 141, 169–182.
Taub, F.B., 1980. Use of continuous culture technique to control nutritional quality. In: Shelef, G., Soeder, C.J. (Eds.), Algae Biomass: Production and Use. Elsevier, Amsterdam, pp. 707–721.
Thinh, L.V., Renaud, S.M., Parry, D.L., 1999. Evaluation of recently isolated Australian tropical microalgae for the enrichment of the dietary value of brine shrimp, *Artemia* nauplii. Aquaculture 170, 161–173.

Tlusty, M.F., Fiore, D.R., Goldstein, J.S., 2005. Use of formulated diets as replacement for *Artemia* in the rearing of juvenile American lobsters (*Homarus americanus*). Aquaculture 250, 781–795.

Tremblay, R., Cartier, S., Miner, P., Pernet, F., Quéré, C., Moal, J., Muzellec, M.L., Mazuret, M., Samain, J.F., 2007. Effect of *Rhodomonas salina* addition to a standard hatchery diet during the early ontogeny of the scallop *Pecten maximus*. Aquaculture 262, 410–418.

Van Stappen, 1996. Use of cysts. In: Sorgeloos, P., Lavens, P. (Eds.), Manual on the Production and Use of Live Food for Aquaculture. Fisheries Technical Paper No. 361. Food and Agriculture Organization of the United Nations, Rome, pp. 107–136.

Woods, C.M., 2003. Effects of varying *Artemia* enrichment on growth and survival of juvenile seahorses, *Hippocampus abdominalis*. Aquaculture 220, 537–548.

Zar, J.H., 1999. Biostatistical Analysis4th Ed. Prentice Hall, Inc, New Jersey. (660 pp).